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June 18, 1998

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BOX PATENT APPLICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

New U.S. Patent Application

Title: NOVEL INSULIN DERIVATIVES HAVING A RAPID ONSET OF ACTION

Inventors: (1) Johann ERTL, (2) Paul HABERMANN,
(3) Karl GEISEN, and (4) Gerhard SEIPKE
Address: (1) Bremthal, Germany, (2) Eppstein, Germany,
(3) Frankfurt, Germany, (4) Hofheim, Germany

Sir:

We enclose the following papers for filing in the United States Patent and Trademark Office in connection with the above patent application.

1. Application - 39 pages, including 1 independent claim and 62 claims total (as amended).
2. Preliminary Amendment.
3. Sequence Listing (7 pages) and computer readable diskette.
4. Certified copy of Germany Application No. 197 26 167.1 filed June 20, 1997.

This application is being filed under the provisions of 37 C.F.R. 1.53(f). Applicants await notification from the Patent and Trademark Office of the time set for filing the Declaration and paying the filing fee of \$1714.00. Please do not charge the filing fee to our Deposit Account without our authorization.

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

The Commissioner is hereby authorized to charge any other fees due under 37 C.F.R. § 1.16 or § 1.17 during the pendency of this application to our Deposit Account No. 06-0916.

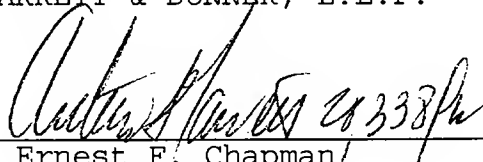
Applicants claim the right to priority based on Germany Application No. 197 26 167.1 filed June 20, 1997.

Please accord this application a serial number and filing date.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By


Ernest F. Chapman/
Reg. No. 25,961

02481.1597/pa
Enclosures

02481.1597/pa

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
Johann ERTL et al.)
Serial No.: TBA) Group Art Unit: TBA
Filed: TBA) Examiner: TBA
For: NOVEL INSULIN DERIVATIVES HAVING)
A RAPID ONSET OF ACTION)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to examination of this application on the merits, Applicants request that the subject application be amended as follows:

IN THE SPECIFICATION:

On page 26, line 1, delete "GLUCOSE-CLAMP BEIM NÜCHTERNEN HUND MIT SCHNELL WIRKSAMEN INSULINDERIVATEN" and insert therefor the following:

--GLUCOSE CLAMP IN FASTING DOGS WITH RAPIDLY ACTING INSULIN
DERIVATIVES--

On page 26, line 2, delete "Dosis: 1 X 0.3 IU/kg s.c. bei t₀" and insert therefor --Dose:

1 x 0.3 IU/kgs.c. at t₀--.

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On page 26, along the lefthand margin, delete "Glucoseinfusions-Rate (mg*kg⁻¹*min⁻¹)" and insert therefor --Glucose infusion rate (mg.kg⁻¹.min⁻¹)--.

On page 26, in the vertical space between the sets of graphs, delete "Blutglucose (mmol/l)" and insert therefor --Blood glucose (mmol/l)--.

On page 26, below the graphs and above "Key:", delete the two recitations of "Minuten" and insert therefor --Minutes--.

On page 26, last seven lines, delete "Key:...Minutes".

Please insert the SEQUENCE LISTING contained in the attached sheets after page 26 and before page 27, i.e., before the claims.

IN THE CLAIMS:

Please cancel claims 37-40, and rewrite as, respectively, new claims 63-66.

Please cancel claim 59, and rewrite as new claim 67.

Please amend the claims as follows:

Page 27, line 1, delete "Patent claims" and insert therefor --We claim:--.

Claim 1, lines 7-11, after "acidic amino acid residue" delete ", it optionally being possible...B chain to be absent".

Claim 8, line 31, "one or more of claims 1 to 7" and insert therefor --claim 1--.

Claim 9, line 3, delete "one or more of claims 1 to 8" and insert therefor --claim 1--.

Claim 13, line 20, delete "one or more of claims 1 to 12" and insert therefor --claim 1--.

Claim 14, line 26, delete "one or more of claims 1 to 13" and insert therefor --claim 1--.

Claim 17, line 9, delete "one or more of claims 1 to 13" and insert therefor --claim 1--.

Claim 26, line 15, delete "one or more of claims 1 to 25" and insert therefor --claim 1--.

Claim 32, line 26, delete "one or more of claims 1 to 30" and insert therefor --claim 1--.

Claim 41, line 15, delete "claim 37" and insert therefor --claim 63--.

Claim 42, line 17, delete "claim 38" and insert therefor --claim 64--.

Claim 43, line 19, delete "claim 39" and insert therefor --claim 65--.

Claim 44, line 21, delete "claim 40" and insert therefor --claim 66--.

Claim 53, line 27, delete "one of claims 49 to 52" and insert therefor --claim 49--.

Claim 54, lines 30-31, delete "one or more of claims 1 to 30" and insert therefor
--claim 1--.

Claim 58, line 14, delete "one of claims 54 to 56" and insert therefor --claim 54--.

Please add the following new claims:

60. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 1, wherein asparagine (Asn) in position 21 of the A chain is replaced by Asp, Gly, Ser, Thr or Ala.

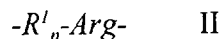
61. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 1, wherein phenylalanine (Phe) in position B1 of the B chain is absent.

62. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 1, wherein the amino acid residue in position B30 of the B chain is absent.

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63. A process for the preparation of an insulin derivative as claimed in claim 26, comprising the construction of a replicable, expression vehicle which contains a DNA sequence which codes for a precursor of the insulin derivative, in which the amino acid residue in position A1 of the A chain is linked to the amino acid residue B30 of the B chain via a peptide chain of the formula II



in which R^I_n is a peptide chain having n amino acid residues and n is an integer from 0 to 34, and the B chain is prologed in position B1 by a peptide chain of the formula III



in which R^2_m is a peptide chain having m amino acid residues, m is an integer from 0 to 40 and p is 0, 1 or 2, expression in a host cell and release of the insulin derivative from its precursor using chemical and/or enzymatic methods, wherein the precursor of the insulin derivative has the sequence

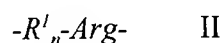
Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg Phe Val Lys Gln
His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr Arg Arg Glu Ala
Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly
Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr
Gln Leu Glu Asn Tyr Cys Asp

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(SEQ ID No.: 11).

64. A process for the preparation of an insulin derivative as claimed in claim 28, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin derivative, in which the amino acid residue in position A1 of the A chain is linked to the amino acid residue B30 of the B chain via a peptide chain of the formula II



in which R^1_n is a peptide chain having n amino acid residues and n is an integer from 0 to 34, and the B chain is prologed in position B1 by a peptide chain of the formula III



in which R^2_m is a peptide chain having m amino acid residues, m is an integer from 0 to 40 and p is 0, 1 or 2, expression in a host cell and release of the insulin derivative from its precursor using chemical and/or enzymatic methods, wherein the precursor of the insulin derivative has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Glu Thr

Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly

Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly

Ser Leu Gln Lys Arg

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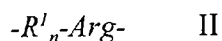
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Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln

Leu Glu Asn Tyr Cys Asn

(SEQ ID NO.: 6).

65. A process for the preparation of an insulin derivative as claimed in claim 30, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin derivative, in which the amino acid residue in position A1 of the A chain is linked to the amino acid residue B30 of the B chain via a peptide chain of the formula II



in which R'_n is a peptide chain having n amino acid residues and n is an integer from 0 to 34, and the B chain is prologed in position B1 by a peptide chain of the formula III



in which R^2_m is a peptide chain having m amino acid residues, m is an integer from 0 to 40 and p is 0, 1 or 2, expression in a host cell and release of the insulin derivative from its precursor using chemical and/or enzymatic methods, wherein the precursor of the insulin derivative has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Ile Pro Lys Thr

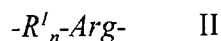
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Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly
 Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly
 Ser Leu Gln Lys Arg
 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
 Leu Glu Asn Tyr Cys Asn

(SEQ ID NO.: 8).

66. A process for the preparation of an insulin derivative as claimed in claim 31, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin derivative, in which the amino acid residue in position A1 of the A chain is linked to the amino acid residue B30 of the B chain via a peptide chain of the formula II



in which R^1_n is a peptide chain having n amino acid residues and n is an integer from 0 to 34, and the B chain is prologed in position B1 by a peptide chain of the formula III



in which R^2_m is a peptide chain having m amino acid residues, m is an integer from 0 to 40 and p is 0, 1 or 2, expression in a host cell and release of the insulin derivative from its precursor using chemical and/or enzymatic methods, wherein the precursor of the insulin derivative has the sequence

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Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg
Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr
Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly
Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly
Ser Leu Gln Lys Arg
Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
Leu Glu Asn Tyr Cys Asn

(SEQ ID NO.: 7).

67. A method for the treatment of diabetes mellitus comprising administering an effective amount of the pharmaceutical preparation of claim 54.

REMARKS

In this amendment, Applicants have amended the specification to insert the English equivalent of German words and have inserted the Sequence Listing in accordance with 37 C.F.R. §§ 1.821-1.825. Additionally, Applicants have amended the claims to remove multiple dependency, to separately claim optional subject matter previously listed in claim 1, to incorporate subject matter into claims previously dependent on more than one claim, and to modify a "use" claim to a "method of treatment" claim. No new matter has been added.

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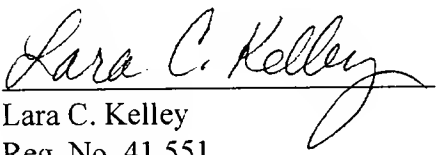
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A computer readable form and paper form of the Sequence Listing is enclosed to be filed with the application. I hereby state that the content of the paper copy and the computer readable copy of the Sequence Listing submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same. I hereby state that the paper copy and the computer readable form copy introduce no new matter and conform with standard Patent and Trademark Office requirements for the Sequence Listing in accordance with 37 C.F.R. §§ 1.821-1.825.

If there are any fees are due in connection with the filing of this amendment, please charge the fees to our Deposit Account No. 06-0916. If a fee is required for an extension of time under 37 C.F.R. 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: 
Lara C. Kelley
Reg. No. 41,551

Dated: June 17, 1998

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Description

5 Novel insulin derivatives having a rapid onset of action

The present invention relates to insulin derivatives which, in comparison to human insulin, have an accelerated onset of action, to a process for their preparation and to their use, in particular in pharmaceutical preparations
10 for the treatment of diabetes mellitus.

Approximately 120 million people worldwide suffer from diabetes mellitus. Among these are approximately 12 million type I diabetics, for whom the administration of insulin is the only therapy possible at present. The
15 affected people are assigned insulin injections, as a rule several times daily, for life. Although type II diabetes, from which approximately 100 million people suffer, is not fundamentally accompanied by an insulin deficiency, in a large number of cases, however, treatment with insulin is regarded as the most favorable or only possible form of therapy.

20 With increasing length of the disease, a large number of the patients suffer from so-called diabetic late complications. These are essentially micro- and macrovascular damage, which depending on the type and extent, result in kidney failure, blindness, loss of extremities or an increased risk
25 of heart/circulation disorders.

As a cause, chronically increased blood glucose levels are primarily held responsible, since even with careful adjustment of the insulin therapy a normal blood glucose profile, such as would correspond to physiological
30 regulation, is not achieved (Ward, J. D. (1989) British Medical Bulletin **45**, 111-126; Drury, P.L. et al. (1989) British Medical Bulletin **45**, 127-147; Kohner, E.M. (1989) British Medical Bulletin **45**, 148-173)

In healthy people, insulin secretion is closely dependent on the glucose concentration of the blood. Increased glucose levels, such as occur after meals, are rapidly compensated by an increased release of insulin. In the fasting state, the plasma insulin level falls to a basal value, which is sufficient to guarantee a continuous supply of insulin-sensitive organs and tissue with glucose. An optimization of the therapy, the so-called intensified insulin therapy, is today primarily aimed at keeping variations in the blood glucose concentration, especially deviations upward, as low as possible (Bolli, G. B. (1989) *Diabetes Res. Clin. Pract.* **6**, p. 3 - p. 16; Berger, M. (1989) *Diabetes Res. Clin. Pract.* **6**, p. 25 - p. 32). This leads to a significant decrease in the occurrence and the progression of diabetic late damage (The Diabetes Control and Complications Trial Research Group (1993) *N. Engl. J. Med.* **329**, 977-986).

From the physiology of insulin secretion, it can be deduced that for an improved, intensified insulin therapy using subcutaneously administered preparations, two insulin preparations having different pharmacodynamics are needed. To compensate the blood glucose rise after meals, the insulin must flow in rapidly and must only act for a few hours. For the basal supply, in particular in the night, a preparation should be available which acts for a long time, has no pronounced maximum and only infuses very slowly.

The preparations based on human and animal insulins only fulfill the demands of an intensified insulin therapy, however, in a restricted manner. After subcutaneous administration, rapidly acting insulins (unmodified insulins) pass too slowly into the blood and to the site of action and have too long an overall duration of action. The result is that the postprandial glucose levels are too high and the blood glucose begins to fall severely several hours after the meal (Kang, S. et al. (1991) *Diabetes Care* **14**, 142-148; Home, P.J. et al. (1989) *British Medical Bulletin* **45**, 92-110; Bolli, G. B. (1989) *Diabetes Res. Clin. Pract.* **6**, p. 3 - p. 16). The available basal

insulins in turn, especially NPH insulins, have too short a duration of action and have a too severely pronounced maximum.

5 Beside the possibility of affecting the profile of action by means of pharmaceutical principles, the alternative presents itself today of designing insulin derivatives, with the aid of genetic engineering, which achieve specific properties such as onset and duration of action solely by means of their structural properties. By the use of suitable insulin derivatives, a significantly better adjustment of the blood glucose more closely adapted to the
10 natural conditions could therefore be achieved.

Insulin derivatives having an accelerated onset of action are described in EP 0 214 826, EP 0 375 437 and EP 0 678 522. EP 0 214 826 relates, inter alia, to substitutions of B27 and B28, but not in combination with the
15 substitution of B3. EP 0 678 522 describes insulin derivatives which have various amino acids, preferably proline, but not glutamic acid, in the position B29. EP 0 375 437 encompasses insulin derivatives having lysine or arginine in B28, which can optionally be additionally modified in B3 and/or A21.

20 EP 0 419 504 discloses insulin derivatives which are protected against chemical modifications by changing asparagine in B3 and at least one further amino acid in the positions A5, A15, A18 or A21. Combinations with modifications in positions B27, B28 or B29 are, however, not described. An
25 indication that these compounds have modified pharmacodynamics resulting in a more rapid onset of action is not given.

WO 92/00321 describes insulin derivatives in which at least one amino acid of the positions B1-B6 is replaced by lysine or arginine. According to
30 WO 92/00321, insulins of this type have a prolonged action. Combinations with modifications of the positions B27, 28, 29, however, are not disclosed.

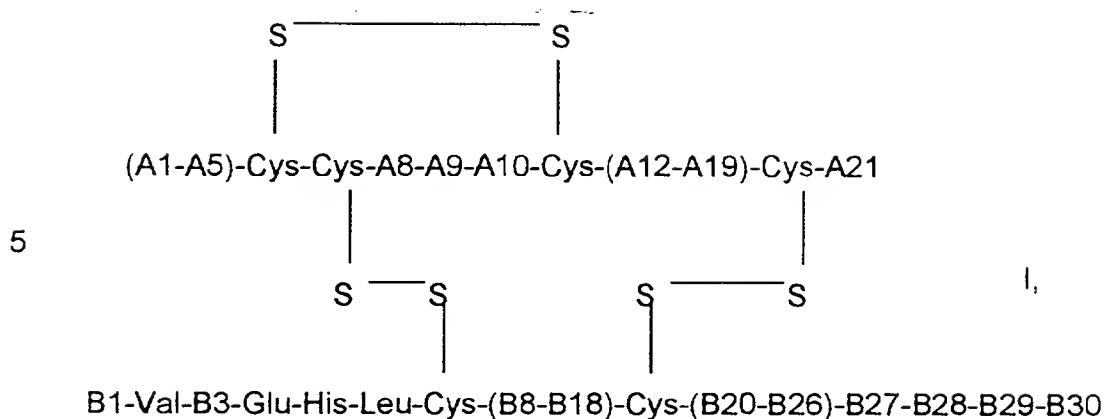
The object of the present invention is to prepare insulin derivatives which after administration, in particular after subcutaneous administration, have an onset of action which is accelerated in comparison with human insulin.

- 5 Insulin derivatives are derivatives of naturally occurring insulins, namely human insulin (see SEQ ID NO 1 = A chain of human insulin; see SEQ ID NO 2 = B chain of human insulin, sequence listing) or animal insulins which differ from the corresponding, otherwise identical naturally occurring insulin by substitution of at least one naturally occurring amino acid
10 residue and/or addition of at least one amino acid residue and/or organic residue.

- It is further an object of the present invention to provide a process for the preparation of the insulin derivatives having the property mentioned, the
15 corresponding intermediates and their precursors.

- The object is achieved by an insulin derivative or a physiologically tolerable salt thereof in which asparagine (Asn) in position B3 of the B chain is replaced by a naturally occurring basic amino acid residue and at
20 least one amino acid residue in the positions B27, B28 or B29 of the B chain is replaced by another naturally occurring amino acid residue, it optionally being possible for asparagine (Asn) in position 21 of the A chain to be replaced by Asp, Gly, Ser, Thr or Ala and for phenylalanine (Phe) in position B1 of the B chain and the amino acid residue in position B30 of
25 the B chain to be absent.

Preferably, the insulin derivative or its physiologically tolerable salt is of formula I



10 in which

(A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin (cf. SEQ ID NO 1) or animal insulin,

15 (A12-A19) are the amino acid residues in the positions A12 to A19 of the A chain of human insulin (cf. SEQ ID NO 1) or animal insulin,

(B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin (cf. SEQ ID NO 2) or animal insulin,

20 (B20-B26) are the amino acid residues in the positions B20 to B26 of the B chain of human insulin (cf. SEQ ID NO 2) or animal insulin,

25 A8, A9, A10 are the amino acid residues in the positions A8, A9 and A10 of the A chain of human insulin (cf. SEQ ID NO 1) or animal insulin,

A21 is Asn, Asp, Gly, Ser, Thr or Ala,

30

B30 is -OH or the amino acid residue in position B30 of the B chain of human insulin (cf. SEQ ID NO 2) or animal insulin,

B1 is a phenylalanine residue (Phe) or a hydrogen atom,
 B3 is a naturally occurring basic amino acid residue,

B27, B28

5 and B29 are the amino acid residues in the positions B27, B28 and B29 of the B chain of human insulin (cf. SEQ ID NO 2) or animal insulin or in each case are another naturally occurring amino acid residue, where at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is
 10 replaced by another naturally occurring amino acid residue.

Of the twenty naturally occurring amino acids which are genetically encodable, the amino acids glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), serine (Ser), threonine (Thr), cysteine (Cys),
 15 methionine (Met), asparagine (Asn), glutamine (Gln), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and proline (Pro) are designated here as neutral amino acids, the amino acids arginine (Arg), lysine (Lys) and histidine (His) are designated as basic amino acids and the amino acids aspartic acid (Asp) and glutamic acid (Glu) are designated as acidic amino
 20 acids.

Preferably, the insulin derivative or its physiologically tolerable salt according to the present invention is a derivative of bovine insulin, porcine insulin or human insulin, namely an insulin derivative or a physiologically
 25 tolerable salt thereof of the formula I, which is distinguished in that

A8 is alanine (Ala),
 A9 is serine (Ser),
 A10 is valine (Val) and
 30 B30 is alanine (Ala) (amino acid residues A8 to A10 and B30 of bovine insulin),

A8 is threonine (Thr),

A9 is serine (Ser) and

A10 is isoleucine (Ile) (amino acid residues A8 to A10 of the insulins of man or pigs), where

B30 is alanine (Ala) (amino acid residue B30 of porcine insulin) or

5 B30 is threonine (Thr) (amino acid residue B30 of human insulin, cf. SEQ ID NO 2).

Particularly preferably, an insulin derivative or a physiologically tolerable salt thereof of the formula I with the amino acid residues A8 to A10 and
10 B30 of human insulin is one which is furthermore distinguished in that

(A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin (cf. SEQ ID NO 1),

15 (A12-A19) are the amino acid residues in the positions A12 to A19 of the A chain of human insulin (cf. SEQ ID NO 1),

(B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin (cf. SEQ ID NO 2) and

(B20-B26) are the amino acid residues in the positions B20 to B26 of the B chain of human insulin (cf. SEQ ID NO 2).

20

Further preferred embodiments of the present invention are an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B1 of the B chain is a phenylalanine residue (Phe) or

25

an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B3 of the B chain is a histidine (His), lysine (Lys) or arginine residue (Arg).

30

Further preferred embodiments of the present invention are an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is replaced by a naturally occurring amino acid

residue which is selected from the group consisting of the neutral or of the acidic amino acids,

an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is a naturally occurring amino acid residue which is selected from the group consisting of isoleucine (Ile), aspartic acid (Asp) and glutamic acid (Glu), preferably wherein at least one of the amino acid residues in the positions B27, B28 of the B chain is replaced by a naturally occurring amino acid residue which is selected from the group consisting of the neutral amino acids, or particularly preferably wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is an isoleucine residue (Ile), or

an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is a naturally occurring amino acid residue which is selected from the group consisting of the acidic amino acids, preferably wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is an aspartic acid residue (Asp), preferably wherein the amino acid residue in position B27 or B28 of the B chain is an aspartic acid residue (Asp), or wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is a glutamic acid residue (Glu).

A preferred embodiment of the present invention is also an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B29 of the B chain is an aspartic acid residue (Asp).

Further preferred embodiments are an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B27 of the B chain is a glutamic acid residue (Glu),

an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B28 of the B chain is a glutamic acid residue (Glu), or

5

an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B29 of the B chain is a glutamic acid residue (Glu).

- 10 Very particularly preferably, an insulin derivative or a physiologically tolerable salt thereof is one which is distinguished in that the B chain has the sequence

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

- 15 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Glu Thr

(SEQ ID NO 3), for example Lys (B3), Glu (B29)-human insulin, or

- 20 an insulin derivative or a physiologically tolerable salt thereof which is distinguished in that the amino acid residue in position B27 of the B chain is an isoleucine residue (Ile), preferably an insulin derivative or a physiologically tolerable salt thereof which is distinguished in that the B chain has the sequence

- 25 Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Ile Pro Lys Thr

- (SEQ ID NO 5), for example Lys (B3), Ile (B27)-human insulin, or
 30 an insulin derivative or a physiologically tolerable salt thereof of the formula I, wherein the amino acid residue in position B28 of the B chain is an isoleucine residue (Ile), preferably an insulin derivative or a

physiologically tolerable salt thereof which is distinguished in that the B chain has the sequence

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

5 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr

(SEQ ID NO 4), for example Lys (B3), Ile (B28)-human insulin.

10 Particularly preferably, an insulin derivative or a physiologically tolerable salt thereof of the formula I, which is distinguished in that the amino acid residue in position B28 of the B chain is an isoleucine residue (Ile) and the amino acid residue in position A21 is an asparagine residue (Asp), is preferably one wherein the A chain has the sequence

15 Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Tyr Gln Leu Glu Asn Tyr Cys Asp (SEQ ID NO.: 9)

and the B chain has the sequence

20 Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Pyr Thr Ile Lys Thr (SEQ ID NO.: 10)

(Lys (B3), Ile (B28), Asp (A21)-human insulin).

25 The insulin derivatives of the formula I can preferably be prepared by genetic engineering.

30 The object set at the outset is accordingly further achieved by a process for the preparation of an insulin derivative or of a physiologically tolerable salt thereof of the formula I, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin derivative in which the amino acid residue in

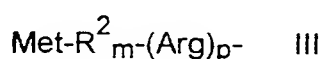
position A1 of the A chain is linked to the amino acid residue B30 of the B chain via a peptide chain of the formula II



5

in which R_n^1 is a peptide chain having n amino acid residues and n is an integer from 0 to 34, and the B chain is prolonged in position B1 by a peptide chain of the formula III

10



15

in which R_m^2 is a peptide chain having m amino acid residues, m is an integer from 0 to 40, preferably from 0 to 9, and p is 0, 1 or 2, where for p = 0 the peptide chain R_m^2 preferably ends with Lys, expression in a host cell and release of the insulin derivative from its precursor using chemical and/or enzymatic methods.

Preferably, the process is one wherein the host cell is a bacterium, particularly preferably one wherein the bacterium is E. coli.

20

Preferably, the process is one wherein the host cell is a yeast, particularly preferably one wherein the yeast is *Saccharomyces cerevisiae*.

25

For the preparation of an insulin derivative having the amino acid sequences SEQ ID NO.: 9 (A chain) and SEQ ID NO.: 10 (B chain), the precursor of this insulin derivative preferably has the sequence

30

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg Phe Val Lys Gln His Leu Cys
Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe
Tyr Thr Ile Lys Thr Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu
Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser

Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr
Gln Leu Glu Asn Tyr Cys Asp (SEQ ID NO.: 11),

a Lys (B3), Ile (B28), Asp (A21)-preproinsulin.

5

For the preparation of an insulin derivative having the amino acid
sequence SEQ ID NO 3, the precursor of this insulin derivative preferably
has the sequence

10 Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Glu Thr

Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly

Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly

15 Ser Leu Gln Lys Arg

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln

Leu Glu Asn Tyr Cys Asn

(Lys (B3), Glu (B29)-preproinsulin)

20 (SEQ ID NO 6).

For the preparation of an insulin derivative having the amino acid
sequence SEQ ID NO 5, the precursor of this insulin derivative preferably
has the sequence

25

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Ile Pro Lys Thr

Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly

Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly

Ser Leu Gln Lys Arg

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln

Leu Glu Asn Tyr Cys Asn

5 (Lys (B3), Ile (B27))-preproinsulin)

(SEQ ID NO 8).

10 For the preparation of an insulin derivative having the amino acid sequence SEQ ID NO 4, the precursor of this insulin derivative preferably has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

15 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr

Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly

Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly

Ser Leu Gln Lys Arg

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln

20 Leu Glu Asn Tyr Cys Asn

(Lys (B3), Ile (B28))-preproinsulin)

(SEQ ID NO 7).

25 The present invention accordingly also relates to said precursors of the preferred insulin derivatives, namely the peptides having the sequence numbers SEQ ID NO.: 11, SEQ ID NO 6, SEQ ID NO 7 and SEQ ID NO 8, the DNA sequences which code for said precursors, the expression vehicles which comprise these DNA sequences and the host cells which
30 are transformed using these expression vehicles.

The insulin derivatives of the formula I are mainly prepared by genetic engineering by means of site-directed mutagenesis according to standard methods.

5

To do this, a gene structure coding for the desired insulin derivative of the formula I is constructed and expressed in a host cell - preferably in a bacterium such as *E. coli* or a yeast, in particular *Saccharomyces cerevisiae* - and - if the gene structure codes for a fusion protein - the
 10 insulin derivative of the formula I is released from the fusion protein; analogous methods are described, for example, in EP-A-0 211 299, EP-A-0 227 938, EP-A-0 229 998, EP-A-0 286 956 and the DE patent application P 38 21 159.

15 The removal of the fusion protein component can be carried out chemically by cell disruption by means of cyanogen halide (see EP-A-0 180 920).

In the preparation by means of a preproinsulin precursor which has a fusion protein component (presequence) according to US 5,358,857, the
 20 removal of the fusion protein component takes place in a later stage together with the removal of the C peptide.

The insulin precursor is then subjected to oxidative sulfitolysis according to the method described, for example, by R.C. Marshall and A.S. Inglis in
 25 "Practical Protein Chemistry - A Handbook" (Publisher A. Darbre) 1986, pages 49 - 53 and then renatured in the presence of a thiol with formation of the correct disulfide bridges, e.g. according to the method described by G.H. Dixon and A.C. Wardlow in *Nature* (1960), pages 721 - 724.

The insulin precursors, however, can also be folded directly
 30 (EP-A-0 600 372; EP-A-0 668 292).

The C peptide is removed by means of tryptic cleavage - e.g. according to the method of Kemmler et al., *J.B.C.* (1971), pages 6786 - 6791 and the

insulin derivative of the formula I is purified by means of known techniques such as chromatography - e.g. EP-A-0 305 760 - and crystallization.

5 If n in formula II is 0, tryptic cleavage serves to sever the peptide bond between A and B chains.

In this process, the B chain C terminal ends with arginine or two arginine residues. These can be removed enzymatically by means of carboxypeptidase B.

10

The insulin derivatives according to the invention have full biological activity. This was shown by intravenous administration to rabbits and the blood glucose fall resulting therefrom (Examples 5 and 6).

15 The more rapid onset of action after subcutaneous administration was shown in fasting dogs using the euglycemic clamp technique (Example 7). 0.3 IU/kg was administered. The reference preparation was human insulin. In the clamp technique, the blood glucose value is measured at short time intervals after insulin injection and exactly the amount of glucose to
 20 compensate the fall is infused. This has the advantage that no counter-regulation occurs with the animals, as would be the case with a severe fall in the blood glucose after the administration of insulin. The amount and the time course of the infused glucose characterize the action of the insulin. Lys(B3), Glu(B29)- (SEQ ID NO 3) and Lys(B3), Ile(B28)- (SEQ ID NO 4)
 25 insulin have a clearly more rapid onset of action than human insulin. The maximum action (glucose infusion rate) is achieved after 100 minutes with human insulin, after 80 minutes, however, with Lys(B3), Glu(B29)-insulin (SEQ ID NO 3) and already after 60 minutes with Lys(B3)-, Ile(B28)-insulin (SEQ ID NO 4). Therefore these analogs, when they are injected shortly
 30 before a meal, should compensate the postprandial rise in the blood glucose better than human insulin.

The insulin derivatives described are therefore suitable both for the therapy of type I and of type II diabetes mellitus, preferably in combination with a basal insulin.

- 5 The present invention therefore also relates to the use of the insulin derivative and/or its physiologically tolerable salt of the formula I for the production of a pharmaceutical preparation which has an insulin activity with a rapid onset of action.
- 10 A suitable carrier medium which is physiologically acceptable and compatible with the insulin derivative is a sterile aqueous solution which is made isotonic with blood in the customary manner, e.g. by means of glycerol, sodium chloride, glucose, and in addition contains one of the customary preservatives, e.g. phenol, m-cresol or p-hydroxybenzoic acid
- 15 ester. The carrier medium can additionally contain a buffer substance, e.g. sodium acetate, sodium citrate, sodium phosphate. For adjustment of the pH, dilute acids (typically HCl) or alkalis (typically NaOH) are used. The preparation can furthermore contain zinc ions.
- 20 The insulin derivatives can be employed in the pharmaceutical preparations even in the form of their physiologically tolerable salts, as alkali metal or as ammonium salts. Any desired proportion of one or more insulin derivatives of the formula I or an insulin derivative of the formula I can be present in a mixture of other of these insulin derivatives
- 25 independently of one another in each case in dissolved, amorphous and/or crystalline form.

- It is sometimes advantageous to add to the preparation according to the invention a suitable amount of a suitable stabilizer which prevents the
- 30 precipitation of protein under thermomechanical stress on contact with various materials. Such stabilizers are disclosed, for example, in EP-A-18609, DE-A 32 40 177 or in WO-83/00288.

The present invention further relates to a pharmaceutical preparation which comprises at least one insulin derivative and/or a physiologically tolerable salt thereof of the formula I, preferably in dissolved, amorphous and/or crystalline form.

5

The insulin derivatives according to the invention have a rapid onset of action. In practical insulin therapy, it is customary under certain circumstances to mix rapid-acting insulins with preparations which contain a depot auxiliary (e.g. NPH insulin). Depending on the composition, preparations result from this whose profiles of action correspond to the superimposed individual profiles provided the individual components in the mixture are stable and are not mutually affected. When mixing an insulin derivative with human NPH insulin, however, it is to be expected that, particularly on long-term storage, an exchange takes place between the dissolved derivative and the crystalline NPH insulin. As a result of this both the pharmacodynamics of the depot insulin and those of the dissolved rapidly acting insulin are changed in an unforeseeable manner. In order to avoid this, it is sensible to prepare the rapidly acting derivative using a depot auxiliary - for example as NPH insulin. This depot form of the insulin derivative can then be mixed as desired with the dissolved rapidly acting form without the composition of one or the other form changing in the course of storage due to exchange.

Although the invention in essence relates to rapidly acting insulin derivatives, it accordingly, however, also comprises the possibility of preparing derivatives of this type as a depot form for the purpose of miscibility, the depot auxiliary preferably being protamine sulfate and the insulin derivative and/or its physiologically tolerable salt being present with the protamine sulfate in a cocrystallizate.

30

The present invention further relates to an injectable solution which comprises the pharmaceutical preparations described above in dissolved form.

Examples

Example 1: Construction of Lys (B3)-proinsulin as a starting point for
5 the plasmids relevant to the invention corresponding to
Examples 2-4

US 5,358,857 describes the vector pINT 90d and the PCR primers Tir and
10 Insu 11. These components serve as starting materials for the construction
of a plasmid pINT 125d, which codes for the desired Lys (B3)-proinsulin.

Additionally, the primers Insu 35 having the sequence

15 5' TTT GTG AAG CAG CAC CTG 3'

and Insu 36 having the sequence

20 5' CAG GTG CTG CTT CAC AAA 3'

are synthesized.

A PCR reaction is carried out using the primers Tir and Insu 36 and a
second reaction is carried out using the primers Insu 11 and Insu 35. The
25 template used for this is pINT 90d DNA.

The products of the two PCR reactions are partially complementary, such
that when they are combined in a third PCR reaction with the primers Tir
and Insu 11 they afford a fragment which codes for a proinsulin variant
which contains the B chain lysine in position 3. This PCR fragment is
30 precipitated in ethanol for purification, dried and then digested with the
restriction enzymes Nco 1 and Sal 1 according to the instructions of the
manufacturer. The reaction mixture is separated by gel electrophoresis
and the desired Nco 1/Sal 1 fragment is isolated.

The application cited describes a plasmid pINT 91d which codes for a mini-proinsulin. If the sequence coding for mini-proinsulin is excised by means of Nco 1 and Sal 1 and the residual plasmid DNA is isolated, this residual plasmid DNA can be reacted with the shown Nco 1/Sal 1 PCR fragment in a T 4 ligase reaction to give the plasmid pINT 125d. This is transformed by E. coli K12, replicated therein and reisolated. After verification of the plasmid structure by means of DNA sequence and restriction analysis, pINT 125d DNA is used as template DNA for the introduction of further mutations into this proinsulin variant.

Example 2: Construction of Lys (B3) Glu (B29)-proinsulin

For the preparation of the mutein, the primers 329a having the sequence

5' TTC TAC ACA CCC GAG ACC CGC GGC ATC G - 3'

and 329b having the sequence

5' GCC GCG GGT CTC GGG TGT GTA GAA GAA GC 3'

are synthesized.

The template used is DNA of the plasmids pINT125d and pINT91d.

Primer 329a is reacted with the primer Insu 11 on the template pINT91d and primer 329b is reacted with Tir (see above example) on the template pINT125d in a PCR reaction. Since both PCR products are partially complementary, they can be combined in a direct PCR reaction and reacted again with the primers Tir and Insu 11. A DNA fragment results which codes for the desired mutein. This fragment is double-digested using the restriction enzymes Nco 1 and Sal 1 and the resulting Nco 1/Sal 1 fragment is inserted into the pINT 91d residual plasmid DNA in a T4 ligase reaction.

The plasmid pINT 329 results, which after amplification in E. coli K12 by means of restriction and DNA sequence analysis is verified with respect to the desired structure.

5

The proinsulin derivative encoded by the plasmid is characterized by the two amino acid replacements and a C-bonding member which consists of the amino acid arginine.

10 Example 3: Construction of Lys (B3) Ile (B27)-proinsulin

The construction is carried out according to the preceding example using the primer pairs

15 KB3 JB 27A

5' TTC TAC ATC CCC AAG ACC CGC CG 3'

and Insu 11

20

and also

K B3 J 27B

5' CTT GGG GAT GTA GAA GAA GCC TCG 3'

25

and Tir.

The template used in both PCR reactions is DNA of the plasmid pINT125d. The PCR products of both reactions are combined in a third reaction, as described in Example 1, and the product is cloned corresponding to the example.

30

The plasmid pINT332 results.

Example 4: Construction of Lys (B3) Ile (B28)-proinsulin

5 The construction is carried out according to Example 3 using the primer pairs:

KB3 JB 28A

5' TAC ACA ATC AAG ACC CGC CGG GAG - 3'

and Insu 11

10

and also

KB J B28B

5' GGT CTT GAT TGT GTA GAA GAA GCC TCG - 3'

15

and Tir.

The plasmid pINT 333 results.

Expression of the constructed insulin variants

20

The plasmids pINT 329, 332 and 333 are each transformed by way of example by E. coli K12 W3110. Recombinant bacteria which contain plasmids which encode the respective variants are then fermented according to Example 4 of the US patent having the patent number
25 5227293 and the desired raw material for the production of the respective insulin variants is thus produced.

Example 5: Construction of Lys (B3), Ile (B28), Asp (A21)-proinsulin

30 Construction is carried out as in Example 3. Instead of pINT125d, however, the template serving for the PCR reaction is DNA of the plasmid pINT333, which was constructed in Example 4. The following primer pair is used here:

P-pint 365

5'-TTTTTGTCTGACTATTAGTCGCAGTAGTTCTACCAGCTG-3'

and Tir.

5 The plasmid pINT365 results.

Example 6: Biological activity of Lys(B3),Glu(B29)-insulin after intravenous administration to rabbits

Time [h]	Human insulin	Lys(B3),Glu(B29)-insulin
0	100	100
0.25	89.17	89.47
0.5	67.56	58.32
0.75	73.24	66.59
1	73.13	68.21
1.5	78.12	71.95
2	89.47	80.88
3	107.01	94.2
4	104.55	99.78

10

8 rabbits received the indicated insulins intravenously (0.2 IU/kg). In the course of the following four hours, the blood glucose concentration was determined at the times indicated and calculated in % of the starting value at time 0. The mean values show no significant differences in the biological activity between human insulin and Lys(B3),Glu(B29)-insulin.

15

Example 7: Biological activity of Lys(B3),Ile(B27)- and Lys(B3),Ile(B28)-insulin after intravenous administration to rabbits

20

6 rabbits received the indicated insulins intravenously (0.2 IU/kg). In the course of the following four hours, the blood glucose concentration was

determined at the times indicated and calculated in % of the starting value at time 0. The mean values show no significant differences in the biological activity between human insulin, Lys(B3),Ile(B27)- and Lys(B3),Ile(B28)-insulin.

5

Time [h]	H insulin	Lys(B3),Ile(B27)-insulin	Lys(B3),Ile(B28)-insulin
0	100	100	100
0.33	67.8	62.6	63.3
0.66	54.9	60.6	55.8
1	55.2	66.8	59.3
1.5	63	79.2	66.7
2	77.8	90.9	81.6
3	91.5	96.3	97.2
4	99.5	96	101.6

Example 8: Pharmacodynamics of Lys(B3),Glu(B29)-insulin and Lys(B3),Ile(B28)-insulin after subcutaneous administration to dogs

10

Four dogs in each case received subcutaneous injections of the indicated insulins (0.3 IU/kg). The blood glucose was kept at 3.7 to 4 mmol/l by continuous infusion of glucose. The mean glucose infusion rate \pm SEM from the time of injection ($t = 0$) through 240 minutes is shown.

15

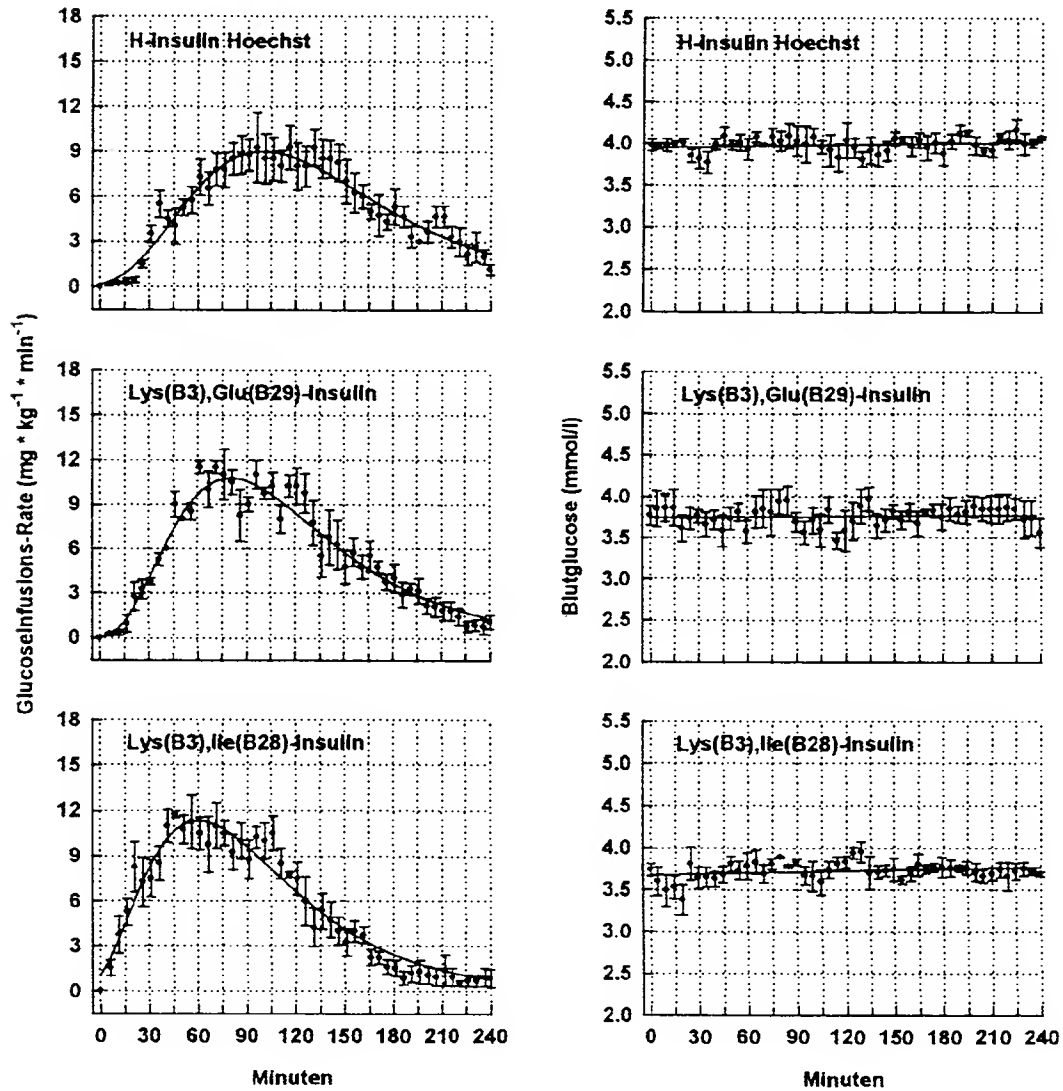
GLUCOSE CLAMP IN FASTING DOGS WITH RAPIDLY ACTING INSULIN DERIVATIVES

Characteristics of the glucose infusion profiles

Dose: 1 x 0.3 IU/kg s.c. at t_0 (n = 4)

Preparation	Increment phase		t_{max} (min)	Decrement phase	
	Inflection point (min)	Slope of the inflection point		Inflection point (min)	Slope of the inflection point
H. insulin, Hoechst	43	0.144	100	156	- 0.065
Lys(B3),Glu(B29) insulin	33	0.227	80	127	- 0.091
Lys(B3),Ile(B28) insulin	16	0.267	60	104	- 0.102

GLUCOSE-CLAMP BEIM NÜCHTERNEN HUND MIT SCHNELL WIRKSAMEN INSULINDERIVATEN

Dosis: $1 \times 0.3 \text{ IU/kg s.c. bei } t_0$ (mean \pm sem, n = 4)

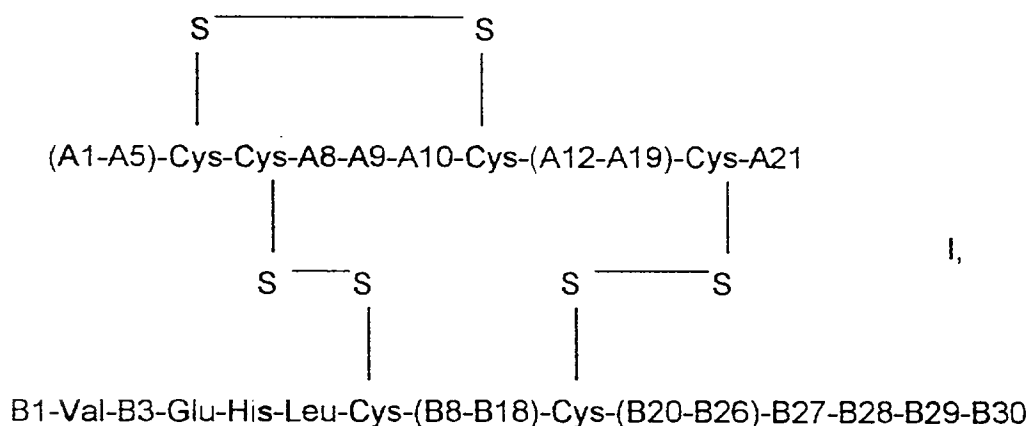
Key:

- 1 GLUCOSE CLAMP IN FASTING DOGS WITH RAPIDLY ACTING INSULIN DERIVATIVES
- 2 Dose: $1 \times 0.3 \text{ IU/kg s.c. at } t_0$
- 3 H-insulin, Hoechst
- 4 Glucose infusion rate (mg \cdot kg $^{-1}$ \cdot min $^{-1}$)
- 5 Minutes

Patent claims

1. An insulin derivative or a physiologically tolerable salt thereof, in which asparagine (Asn) in position B3 of the B chain is replaced by a naturally occurring basic amino acid residue and at least one amino acid residue in the positions B27, B28 or B29 of the B chain is replaced by another naturally occurring neutral or acidic amino acid residue, it optionally being possible for asparagine (Asn) in position 21 of the A chain to be replaced by Asp, Gly, Ser, Thr or Ala and for phenylalanine (Phe) in position B1 of the B chain and the amino acid residue in position B30 of the B chain to be absent.

2. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 1, of formula I



in which

(A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin or animal insulin,

(A12-A19) are the amino acid residues in the positions A12 to A19 of the A chain of human insulin or animal insulin,

A21 is Asn, Asp, Gly, Ser, Thr or Ala,

(B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin or animal insulin,

(B20-B26) are the amino acid residues in the positions B20 to B26 of the B chain of human insulin or animal insulin,

A8, A9, A10 are the amino acid residues in the positions A8, A9 and A10 of the A chain of human insulin or animal insulin,

B30 is -OH or the amino acid residue in position B30 of the B chain of human insulin or animal insulin,

B1 is a phenylalanine residue (Phe) or a hydrogen atom,

B3 is a naturally occurring basic amino acid residue,

B27, B28

and B29 are the amino acid residues in the positions B27, B28 and B29 of the B chain of human insulin or animal insulin or in each case are another naturally occurring amino acid residue, where at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is replaced by another naturally occurring amino acid residue which is selected from the group consisting of the neutral or acidic amino acids.

3. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 2, wherein

A8 is alanine (Ala),

A9 is serine (Ser),

A10 is valine (Val) and

B30 is alanine (Ala).

4. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 2, wherein

A8 is threonine (Thr),

5 A9 is serine (Ser) and

A10 is isoleucine (Ile).

5. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 4, wherein

10 B30 is alanine (Ala).

6. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 4, wherein

B30 is threonine (Thr).

15 7. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 6, wherein

(A1-A5) are the amino acid residues in the positions A1 to A5 of the A chain of human insulin,

20 (A12-A19) are the amino acid residues in the positions A12 to A19 of the A chain of human insulin,

25 (B8-B18) are the amino acid residues in the positions B8 to B18 of the B chain of human insulin and

(B20-B26) are the amino acid residues in the positions B20 to B26 of the B chain of human insulin.

30 8. An insulin derivative or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 7, wherein the amino acid residue in position B1 of the B chain is a phenylalanine residue (Phe).

9. An insulin derivative or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 8, wherein the amino acid residue in position B3 of the B chain is a histidine (His), lysine (Lys) or arginine residue (Arg).

10. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 9, wherein the amino acid residue in position B3 of the B chain is a histidine residue (His).

11. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 9, wherein the amino acid residue in position B3 of the B chain is an arginine residue (Arg).

12. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 9, wherein the amino acid residue in position B3 of the B chain is a lysine residue (Lys).

13. An insulin derivative or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 12, wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is a naturally occurring amino acid residue which is selected from the group consisting of isoleucine (Ile), aspartic acid (Asp) and glutamic acid (Glu).

14. An insulin derivative or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 13, wherein at least one of the amino acid residues in the positions B27, B28 and B29 of the B chain is a naturally occurring amino acid residue which is selected from the group consisting of the acidic amino acids.

15. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 14, wherein at least one of the amino acid residues in the

positions B27, B28 and B29 of the B chain is an aspartic acid residue (Asp).

16. An insulin derivative or a physiologically tolerable salt thereof as
5 claimed in claim 14, wherein at least one of the amino acid residues in the
positions B27, B28 and B29 of the B chain is a glutamic acid residue (Glu).

17. An insulin derivative or a physiologically tolerable salt thereof as
10 claimed in one or more of claims 1 to 13, wherein at least one of the amino
acid residues in the positions B27, B28 of the B chain is replaced by a
naturally occurring amino acid residue which is selected from the group
consisting of the neutral amino acids.

18. An insulin derivative or a physiologically tolerable salt thereof as
15 claimed in claim 17, wherein at least one of the amino acid residues in the
positions B27, B28 and B29 of the B chain is an isoleucine residue (Ile).

19. An insulin derivative or a physiologically tolerable salt thereof as
20 claimed in claim 15, wherein the amino acid residue in position B27 of the
B chain is an aspartic acid residue (Asp).

20. An insulin derivative or a physiologically tolerable salt thereof as
25 claimed in claim 15, wherein the amino acid residue in position B28 of the
B chain is an aspartic acid residue (Asp).

21. An insulin derivative or a physiologically tolerable salt thereof as
claimed in claim 15, wherein the amino acid residue in position B29 of the
B chain is an aspartic acid residue (Asp).

22. An insulin derivative or a physiologically tolerable salt thereof as
30 claimed in claim 16, wherein the amino acid residue in position B27 of the
B chain is a glutamic acid residue (Glu).

23. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 16, wherein the amino acid residue in position B28 of the B chain is a glutamic acid residue (Glu).

5

24. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 16, wherein the amino acid residue in position B29 of the B chain is a glutamic acid residue (Glu).

10

25. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 18, wherein the amino acid residue in position B28 of the B chain is an isoleucine residue (Ile).

15

26. An insulin derivative or a physiologically tolerable salt thereof as claimed in one of more of claims 1 to 25, wherein the amino acid residue in position A21 of the A chain is an asparagine residue (Asp).

20

27. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 26, wherein the A chain has the sequence

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn
Tyr Cys Asp (SEQ ID NO.: 9)

and the B chain has the sequence

25

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val
Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr (SEQ ID NO.: 10).

30

28. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 24, wherein the B chain has the sequence

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Glu Thr

(SEQ ID NO 3).

- 5 29. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 18, wherein the amino acid residue in position B27 of the B chain is an isoleucine residue (Ile).

- 10 30. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 29, wherein the B chain has the sequence

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Ile Pro Lys Thr

- 15 (SEQ ID NO 5).

31. An insulin derivative or a physiologically tolerable salt thereof as claimed in claim 25, wherein the B chain has the sequence

- 20 Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

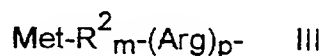
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr

(SEQ ID NO 4).

- 25 32. A process for the preparation of an insulin derivative or of a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 30, comprising the construction of a replicable expression vehicle which contains a DNA sequence which codes for a precursor of the insulin derivative, in which the amino acid residue in position A1 of the A chain is
- 30 linked to the amino acid residue B30 of the B chain via a peptide chain of the formula II



in which R_n^1 is a peptide chain having n amino acid residues and n is an integer from 0 to 34, and the B chain is prolonged in position B1 by a peptide chain of the formula III



in which R_m^2 is a peptide chain having m amino acid residues, m is an integer from 0 to 40 and p is 0, 1 or 2, expression in a host cell and release of the insulin derivative from its precursor using chemical and/or enzymatic methods.

33. The process as claimed in claim 32, wherein the host cell is a bacterium.

34. The process as claimed in claim 33, wherein the bacterium is *E. coli*.

35. The process as claimed in claim 32, wherein the host cell is a yeast.

36. The process as claimed in claim 35, wherein the yeast is *Saccharomyces cerevisiae*.

37. The process as claimed in one of claims 32 to 36 for the preparation of an insulin derivative as claimed in claim 26, wherein the precursor of the insulin derivative has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg Phe Val Lys Gln
His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr Arg Arg Glu Ala

Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly
Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr
Gln Leu Glu Asn Tyr Cys Asp

5

(SEQ ID NO.: 11).

38. The process as claimed in one of claims 32 to 36 for the preparation
of an insulin derivative as claimed in claim 28, wherein the precursor of the
insulin derivative has the sequence

10

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg
Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Glu Thr
Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly
Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly
Ser Leu Gln Lys Arg
Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
Leu Glu Asn Tyr Cys Asn

15
20

(SEQ ID NO 6).

39. The process as claimed in one of claims 32 to 36 for the preparation
of an insulin derivative as claimed in claim 30, wherein the precursor of the
insulin derivative has the sequence

25

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg
Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Ile Pro Lys Thr

Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly
 Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly
 Ser Leu Gln Lys Arg

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln

5 Leu Glu Asn Tyr Cys Asn

(SEQ ID NO 8).

40. The process as claimed in one of claims 32 to 36 for the preparation
 10 of an insulin derivative as claimed in claim 31, wherein the precursor of the
 insulin derivative has the sequence

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu

15 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr
 Arg Arg Glu Ala Glu Asp Pro Gln Val Gly Gln Val Glu Leu Gly
 Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly
 Ser Leu Gln Lys Arg

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln

20 Leu Glu Asn Tyr Cys Asn

(SEQ ID NO 7).

41. A precursor of the insulin derivative as claimed in claim 37.

25 42. A precursor of the insulin derivative as claimed in claim 38.

43. A precursor of the insulin derivative as claimed in claim 39.

44. A precursor of the insulin derivative as claimed in claim 40.

45. A DNA sequence which codes for a precursor of the insulin derivative as claimed in claim 41.

5 46. A DNA sequence which codes for a precursor of the insulin derivative as claimed in claim 42.

47. A DNA sequence which codes for a precursor of the insulin derivative as claimed in claim 43.

10

48. A DNA sequence which codes for a precursor of the insulin derivative as claimed in claim 44.

15

49. An expression vehicle comprising a DNA sequence as claimed in claim 45.

50. An expression vehicle comprising a DNA sequence as claimed in claim 46.

20

51. An expression vehicle comprising a DNA sequence as claimed in claim 47.

52. An expression vehicle comprising a DNA sequence as claimed in claim 48.

25

53. A host cell which is transformed using an expression vehicle as claimed in one of claims 49 to 52.

30

54. A pharmaceutical preparation, which comprises at least one insulin derivative and/or a physiologically tolerable salt thereof as claimed in one or more of claims 1 to 30.

55. A pharmaceutical preparation as claimed in claim 54, which comprises the insulin derivative and/or the physiologically tolerable salt thereof in dissolved, amorphous and/or crystalline form.

5 56. A pharmaceutical preparation as claimed in claim 54, which further comprises a depot auxiliary.

57. A pharmaceutical preparation as claimed in claim 56, wherein the depot auxiliary is protamine sulfate, where the insulin derivative and/or the physiologically tolerable salt thereof is present with the protamine sulfate
10 in a cocrystallizate.

58. An injectable solution having insulin activity, comprising the pharmaceutical preparation as claimed in one of claims 54 to 56 in
15 dissolved form.

59. The use of the insulin derivative and/or its physiologically tolerable salt as claimed in one or more of claims 1 to 31 for the production of a pharmaceutical preparation which has an insulin activity with a rapid onset
20 of action.

Abstract

Novel insulin derivatives having a rapid onset of action

The present invention relates to insulin derivatives which in comparison to human insulin, have an accelerated onset of action, to a process for their preparation and to their use, in particular in pharmaceutical preparations for the treatment of diabetes mellitus.

In particular, the present invention relates to insulin derivatives or physiologically tolerable salts thereof in which asparagine (Asn) in position B3 of the B chain is replaced by a naturally occurring basic amino acid residue and at least one amino acid residue in the positions B27, B28 or B29 of the B chain is replaced by another naturally occurring amino acid residue, it optionally being possible for asparagine (Asn) in position 21 of the A chain to be replaced by Asp, Gly, Ser, Thr or Ala and for phenylalanine (Phe) in position B1 of the B chain and the amino acid residue in position B30 of the B chain to be absent.

2007-01-01

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ertl, Johann
Habermann, Paul
Geisen, Karl
Seipke, Gerhard

(ii) TITLE OF INVENTION: Novel Insulin Derivatives Having A
Rapid Onset of Action

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20005-3315

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Leslie McDonell
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(C) REFERENCE/DOCKET NUMBER: 02481.1597-00000

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(B) TELEFAX: (202) 408-4400

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1 5 10 15

Glu Asn Tyr Cys Asn
20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr
20 25 30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Glu Thr
20 25 30

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Phe	Val	Lys	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr
1				5				10						15	

Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Ile	Lys	Thr
			20				25						30

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Phe	Val	Lys	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr
1				5				10						15	

Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Ile	Pro	Lys	Thr
			20				25						30

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met	Ala	Thr	Thr	Ser	Thr	Gly	Asn	Ser	Ala	Arg	Phe	Val	Lys	Gln	His
1				5					10					15	
Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu
			20					25					30		
Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Glu	Thr	Arg	Arg	Glu	Ala	Glu	Asp	Pro
		35					40					45			
Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu
	50					55					60				
Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln	Lys	Arg	Gly	Ile	Val	Glu
65					70					75					80
Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys
				85					90					95	

Asn

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 97 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met	Ala	Thr	Thr	Ser	Thr	Gly	Asn	Ser	Ala	Arg	Phe	Val	Lys	Gln	His
1				5					10					15	
Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu
			20					25					30		
Arg	Gly	Phe	Phe	Tyr	Thr	Ile	Lys	Thr	Arg	Arg	Glu	Ala	Glu	Asp	Pro
		35					40					45			
Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu
	50					55					60				
Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln	Lys	Arg	Gly	Ile	Val	Glu
65					70					75					80

Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
85 90 95

Asn

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 97 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg Phe Val Lys Gln His
1 5 10 15
Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu
20 25 30
Arg Gly Phe Phe Tyr Ile Pro Lys Thr Arg Arg Glu Ala Glu Asp Pro
35 40 45
Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu
50 55 60
Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu
65 70 75 80
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
85 90 95

Asn

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
 1 5 10 15
 Glu Asn Tyr Cys Asp
 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Phe Val Lys Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
 1 5 10 15
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Ile Lys Thr
 20 25 30

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 97 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Met Ala Thr Thr Ser Thr Gly Asn Ser Ala Arg Phe Val Lys Gln His
 1 5 10 15
 Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu
 20 25 30
 Arg Gly Phe Phe Tyr Thr Ile Lys Thr Arg Arg Glu Ala Glu Asp Pro
 35 40 45
 Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu
 50 55 60

Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu
65 70 75 80

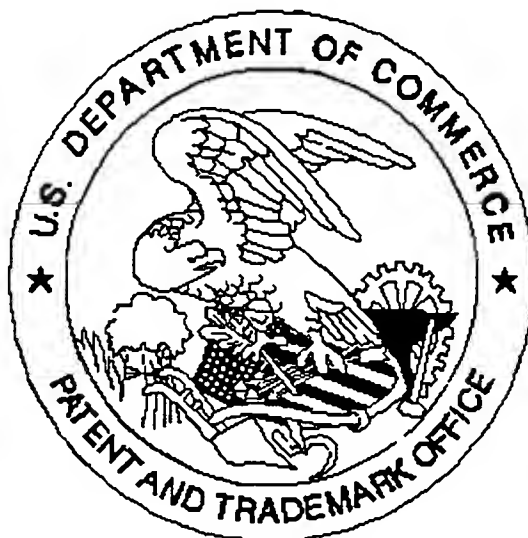
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
85 90 95

Asp

110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960 965 970 975 980 985 990 995

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